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ABSTRACT

An adenovirus encoding the genes for human somatostatin receptor subtype 2 has been constructed and evaluated in human prostate cancer cells with regard to binding of ^{64}Cu-octreotide. In vivo experiments were conducted in scid mice bearing subcutaneous DU-145 or PC-3 cells. AdSSTR2 was injected intratumorally followed 48 h later by an i.v. injection of ^{64}Cu-octreotide. The mice were sacrificed 1 h after peptide injection for biodistribution analysis. In vivo biodistribution studies showed similar uptake of ^{64}Cu-octreotide in both DU-145 and PC-3 tumors after infection with AdSSTR2 (2.5 and 2.7% ID/g, respectively). This uptake was greater than that observed in tumors injected with control adenovirus (1.4 - 1.6% ID/g). Another adenovirus encoding for both SSTR2 and cytosine deaminase (CD) was constructed (AdSSTR2CD) and evaluated in PC-3 and DU-145 cells. This showed that there was good correlation between the expression of SSTR2 and CD after infection with AdSSTR2CD. In addition, CD activity was confirmed using a tritiated 5-fluorocytosine assay.

Table of Contents

Introduction

It is estimated that approximately 37,000 U.S. men died in 1999 from prostate cancer. It is clear that novel treatments for prostate cancer are necessary. Radiolabeled monoclonal antibodies have been used to treat hormone-refractory prostate cancer with limited success. Reasons for these limitations include, bone marrow toxicity from the long serum half-life of the radiolabeled antibody, heterogeneous tumor distribution of the large molecular weight antibody, and low tumor antigen/receptor expression. A strategy to overcome these limitations is to combine peptide radiotherapy with gene therapy. Radiolabeled peptides can overcome problems associated with bone marrow toxicity and tumor penetration due to their small molecular weight, while gene therapy can be used to increase the tumor antigen/receptor expression. Previous studies have shown that an adenovirus encoding for the somatostatin receptor subtype 2 (AdSSTR2) can be used to increase tumor localization of radiolabeled octreotide analogues. **Objective/Hypothesis.** The objective of this proposal is to determine if induction of SSTR2 with AdSSTR2 on human prostate cancer xenografts in mice has a therapeutic effect after targeting with the octreotide analogue, ^{64}Cu-octreotide. **Specific Aims.** SPECIFIC AIM #1. Evaluate the expression of SSTR2 on human prostate cancer cells *in vitro* after infection with AdSSTR2 using radiolabeled octreotide binding and internalization assays. SPECIFIC AIM #2. Evaluate the distribution of radiolabeled octreotide after i.v. injection by non-invasive PET imaging and by gamma counter analysis in nude mice bearing s.c. human prostate cancer xenografts injected with AdSSTR2. SPECIFIC AIM #3. Perform therapy studies in a mouse model of human prostate cancer utilizing the best vector as determined in Specific Aims 1 and 2 and ^{64}Cu-octreotide. **Study Design.** The first aim of the study will evaluate SSTR2 expression on PC-3 and DU-145 human prostate cancer cells *in vitro* after infection with AdSSTR2. These assays will be conducted using ^{64}Cu-octreotide in Scatchard and internalization experiments. The Scatchard analysis will determine the level of SSTR2 expression on the cells and the internalization of SSTR2 is important for the subsequent localization and therapy studies. The PC-3 and DU-145 cells will then be implanted s.c. in athymic nude mice and SSTR2 expression will be determined by ^{64}Cu-octreotide tumor localization following injection of AdSSTR2. These studies will be conducted using PET imaging, tissue counting in a gamma counter and immunohistochemistry. Therapy will be conducted in mice bearing subcutaneous PC-3 and DU-145 tumors following injection of AdSSTR2 and i.v. injections of ^{64}Cu-octreotide. **Relevance.** These studies are directly relevant to improving the treatment of hormone-refractory prostate cancer. Novel therapies are needed for the treatment of this disease and this proposal introduces a new paradigm for its treatment by combining targeted radiolabeled peptide therapy with gene therapy. In addition, this strategy can be used to detect prostate cancer using external PET imaging.

Body

Statement of Work

SPECIFIC AIM #1. Evaluate the expression of SSTR2 on human prostate cancer cells *in vitro* after infection with AdSSTr2 using radiolabeled octreotide binding and internalization assays.

Task1: Months 1-4: Radiolabel octreotide with ^{64}Cu and use the ^{64}Cu-octreotide to determine the level of SSTR2 expression in PC-3 and DU-145 human prostate cancer cells after infection with various amounts of AdSSTR2. This will be done by Scatchard analysis. In addition, internalization of SSTr2 and ^{64}Cu-octreotide will be evaluated.

SPECIFIC AIM #2. Evaluate the distribution of radiolabeled octreotide after i.v. injection by non-invasive PET imaging and by gamma counter analysis in nude mice bearing s.c. human prostate cancer xenografts injected and with AdSSTR2.

Task 1: Months 3-12: Athymic nude mice will be implanted s.c. with PC-3 and DU-145 human prostate cancer cells and injected intratumorally with AdSSTr2 3-5 weeks later. ^{64}Cu-octreotide will then be administered i.v. and the mice imaged using micro PET to determine SSTR2 expression and ^{64}Cu-octreotide distribution.

Task 2: Months 3-12: Athymic nude mice will be implanted s.c. with PC-3 and DU-145 human prostate cancer cells and injected intratumorally with AdSSTR2 3-5 weeks later. ^{64}Cu-octreotide will then be administered i.v. and the mice will be sacrificed to determine SSTr2 expression by immunohistochemistry and counting tissues in a gamma counter. These studies will be complementary to those discussed in Task 1.

Task 3: Months 6-15: Athymic nude mice will be implanted s.c. with PC-3 and DU-145 human prostate cancer cells and injected **i.v.** with AdSSTR2 3-5 weeks later. ^{64}Cu-octreotide will then be administered i.v. and the mice will be sacrificed to determine SSTR2 expression by immunohistochemistry and counting tissues in a gamma counter. These studies will be an initial step towards evaluating this system in the context of hormone-refractory disease. Administration of the vectors i.v. will not be used in therapy studies unless the tumor expression of SSTr2 is at least two-fold greater than expression in the liver.

SPECIFIC AIM #3. Perform therapy studies in a mouse model of human prostate cancer utilizing the best vector as determined in Specific Aims 1 and 2 and ^{64}Cu-octreotide.

Task 1: Months 13-24: Athymic nude mice will be implanted s.c. with PC-3 and DU-145 human prostate cancer cells and injected intratumorally with AdSSTR2 3-5 weeks later. Various therapeutic doses of ^{64}Cu-octreotide will be administered i.v. 2 and 4 days after adenovirus. Tumors will be measured every 3 days to determine if there is any response to treatment. Controls will include an irrelevant adenovirus injection, unlabeled octreotide injections, and no treatment. Toxicity will also be monitored throughout the studies.

Since the previous annual report, the PI has evaluated AdSSTR2 in human prostate cancer xenografts in scid mice and the biodistribution of ^{64}Cu-octreotide was evaluated after intratumoral injection of AdSSTR2. These studies were directly related to Task 2 of Specific Aim 2 and are shown in **Figures 1** and **2.** As can be seen in these figures, the uptake of ^{64}Cu-octreotide in DU-145 and PC-3 tumors injected with AdSSTR2 was greater than control tumors, but less than many normal tissues. Because of this unimpressive uptake, we decided that it was not worth pursuing the goals as stated in Tasks 1 and 3 since these experiments were more stringent than Task 2. Instead, we have started to investigate another approach for treating prostate cancer xenografts which involves the use of an adenoviral vector encoding for both SSTR2 and the cytosine deaminase (CD) enzyme. It is hypothesized that this vector can increase the therapeutic efficacy of SSTR2-directed radiotherapy by CD converting the prodrug 5-fluorocytosine to the toxic and radiosensitizing drug 5-fluorouracil.

Methods

In vivo biodistribution studies. Biodistribution studies were performed in scid mice bearing s.c. PC-3 or DU-145 tumors. The mice were injected s.c. with 1 x 10^7 PC-3 or DU-145 cells (1:1 mixture with matrigel) followed by intratumoral injection with 3 x 10^8 pfu of AdSSTR2 after the tumors were established. Two days after adenoviral injection, the mice were injected i.v. with ^{64}Cu-octreotide (10 μCi) and sacrificed 1 h later. The tissues were then harvested and counted in a gamma counter to determine the biodistribution as the % injected dose per gram of tissue (% ID/g).

Construction of an adenovirus containing SSTR2 and CD. Construction of an adenovirus containing both SSTR2 and bacterial cytosine deaminase (CD) was performed in a manner similar to that previously described. The AdEasy system was used to generate the final adenoviral product containing SSTR2 and CD, both under the control of independent cytomegalovirus (CMV) promoters. First, an extended polylinker was inserted into the cloning vector pShuttle-CMV to create pShuttle-CMVX. Second, subcloning of SSTR2 into pShuttle-CMVX was performed to create pShuttle-SSTR2. PCR amplification of the CMV promoter with artificial restriction sites on the 5' and 3' ends followed by subsequent digestion and insertion into pShuttle-SSTR2 was then performed. Finally, PCR amplification of CD from pACMVCD followed by cloning into the SSTR2-CMV vector was performed. The final product for incorporation into virus was a shuttle vector containing CMV-independently controlled SSTR2 followed by another CMV promoter that controls expression of CD. Incorporation of SSTR2 and CD into the pAdEasy-1 vector was performed as described in the manufacturer's protocol. Homologous recombination between the shuttle vector containing SSTR2 and CD and pAdEasy1 was carried out in *E. coli* upon which recombinant pAdEasy was purified for transfection and subsequent viral production in HEK293 cells.

Correlation of SSTR2 expression to CD activity. A saturating binding curve using membrane preparations of DU-145 and PC-3 cells infected with adenovirus was used to determine the expression of SSTR2 (in fmol/mg) 2 days after adenoviral infection. To determine CD activity, the cells were seeded in T75 flasks and incubated overnight at 37°C. The cells were then infected with adenovirus for 1 h at 37°C at the same MOI as used in the saturation binding assay. One day later, the cells were harvested and plated into 96 well tissue culture plates at 5000 cells/well in 100 μl of complete media. Twenty-four hours later, 100 μl of media containing various concentrations of 5-FC were added to the cells in triplicate and the cells allowed to incubate an additional 5 days at 37°C. The fractional cell survival at each drug concentration was determined using an MTS assay calculated as the ratio of absorbance at 490 nm of cells incubated in the presence versus absence of drug, corrected for background absorbance of media alone. Fractional cell survival data was plotted against the logarithm of drug concentration and IC_{50} values will be determined using the GraphPad Prism software. The correlation between CD activity (IC_{50}) and mHAhSSTR2 (fmol/mg) was then be determined by linear regression.

CD activity can be determined using a tritiated 5-FC conversion assay. CD activity has also been determined using a tritiated conversion assay. In this study, we infected PC-3 cells with AdhSSTR2CD at 100 MOI and prepared lysates 48 h after infection. A protein assay was performed and various amounts of the lysate were incubated with various amounts of 5-FC (0.2 to 100 mM) spiked with $[^3H]$-5-FC. The mixtures were incubated for various times and then an aliquot was applied to a TLC plate to separate $[^3H]$-5-FC from $[^3H]$-5-FU. The conversion of 5-FC to 5-FU was determined using a TLC scanner and the rate of conversion (V) was determined at each 5-FC concentration. The rate limiting velocity at high substrate concentration (V_{max}) was determined by with a Michaelis-Menten plot using GraphPad Prism.

Results

The biodistribution studies demonstrate that SSTR2 is expressed in both DU-145 and PC-3 tumors after intratumoral injection of AdSSTR2. **Figures 1** and **2** show uptake of ^{64}Cu-octreotide in DU-145 and PC-3 tumors that were injected with AdSSTR2 that was greater than uptake in tumors injected with a control adenovirus. DU-145 tumors injected with AdSSTR2 showed 2.5 ± 1.6% ID/g compared to 2.7 ± 0.7% ID/g in PC-3 cells. DU-145 and PC-3 cells injected with a control adenovirus showed 1.4 ± 0.1% ID/g and 1.6 ± 0.2% ID/g respectively.

The correlation between AdSSTR2CD dose (MOI) and SSTR2 expression (fmol/mg) is shown in **Figure 3A**. A significant correlation exists for both DU-145 and PC-3 cells with $p < 0.04$ for both cell lines. Cytosine deaminase activity was determined by addition of 5-FC two days after adenoviral infection to calculate IC_{50} values (μM) determined by MTS assay after a 5 day drug exposure as previously described. **Figure 3B** shows the correlation between AdSSTR2CD dose (MOI) and CD activity as a function of IC_{50} values (μM). Again, a significant correlation is observed with both cell lines ($p < 0.03$). The correlation between SSTR2 expression and CD activity is shown in **Figure 3C**. There is a strong correlation between SSTR2 and CD that is significant for both cell lines ($p < 0.05$). These studies demonstrate the feasibility of using SSTR2 to predict the activity of CD. Studies similar to these will be performed in animal models to show that the relationship still exists.

The results of the tritiated 5-FC conversion assay are shown in **Figure 4**. This shows that the V_{max} for PC-3 cells infected at 100 MOI with AdSSTR2CD is 0.062 nmol/mg/min. This study demonstrates that we can use this assay to determine a V_{max} after infection with a given amount of adenovirus. It will be used to determine CD activity in tumor xenografts infected with various amounts of adenovirus. Thus, we will be able to correlate CD activity (V_{max}) at various adenoviral doses with SSTR2 expression (% ID/g).

Key Research Accomplishments

- Expression of SSTR2 in DU-145 and PC-3 tumor xenografts after intratumoral injection of AdSSTR2 could be detected by binding of ^{64}Cu-octreotide in a biodistribution study.
- A new adenovirus was constructed that contained SSTR2 and CD (AdSSTR2CD)
- Good correlations were observed between SSTR2 expression and CD expression in DU-145 and PC-3 cells after infection with AdSSTR2CD.
- A tritiated 5-FC assay could determine the V_{max} in PC-3 cells after infection with AdSSTR2CD.

Reportable Outcomes

None

Conclusions

These studies demonstrate that AdSSTR2 can infect both DU-145 and PC-3 human prostate cancer cells and result in expression of SSTR2. This was demonstrated through binding of ^{64}Cu-octreotide. However, the binding of ^{64}Cu-octreotide was still less than many normal tissues. Because of this, it was decided that it was not worth pursuing this strategy further in therapy studies. Instead we are pursuing a combination strategy that utilizes an adenovirus that contains both SSTR2 and CD. We have demonstrated a good correlation in expression of these proteins in prostate cancer cells after infection with AdSSTR2CD and demonstrated that we can detect CD activity using a tritiated 5-FC conversion assay.

Appendices

Figure Legend

Figures

Figure Legend

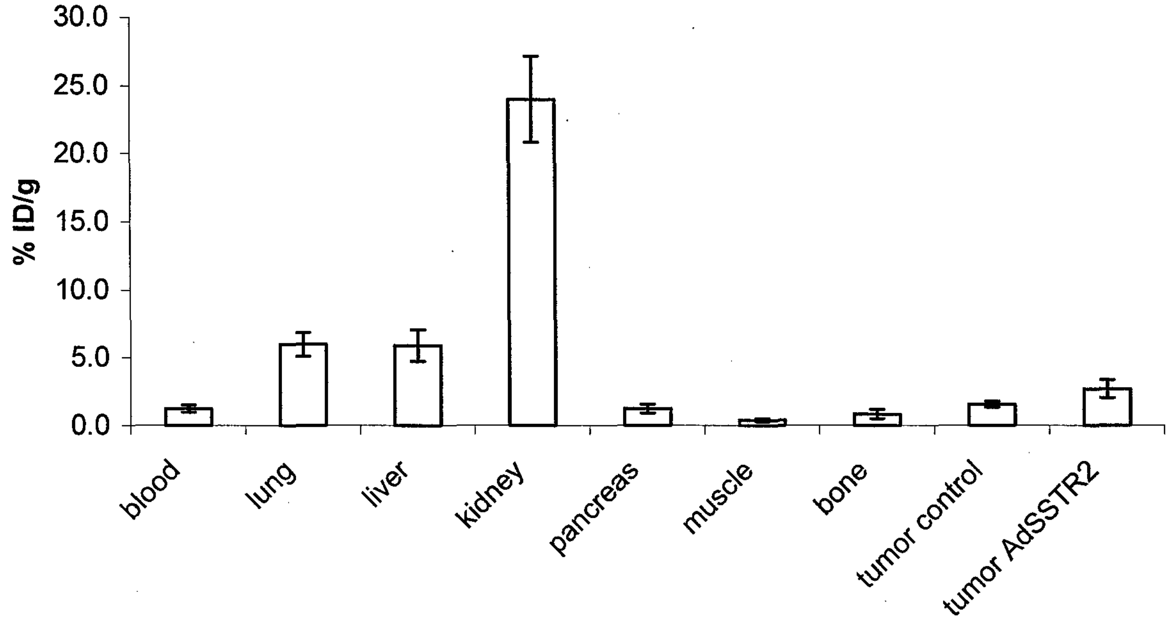
Figure 1. Biodistribution of ^{64}Cu-octreotide in scid mice bearing DU-145 tumors. Tumors were injected intratumorally with 3 x10^8 pfu of AdSSTR2 or a control adenovirus followed by i.v. injection of ^{64}Cu-octreotide 2 days later. The mice were sacrificed 1 h after injection of ^{64}Cu-octreotide and tissues harvested, weighed and counted in a gamma counter. Data represent the mean ± standard deviation (n = 4-6 animals).

Figure 2. Biodistribution of ^{64}Cu-octreotide in scid mice bearing PC-3 tumors. Tumors were injected intratumorally with 3 x10^8 pfu of AdSSTR2 or a control adenovirus followed by i.v. injection of ^{64}Cu-octreotide 2 days later. The mice were sacrificed 1 h after injection of ^{64}Cu-octreotide and tissues harvested, weighed and counted in a gamma counter. Data represent the mean ± standard deviation (n = 4-6 animals).

Figure 3. Correlation of SSTR2 expression with AdSSTR2CD dose for PC-3 (10, 30, 100, 200 MOI) and DU-145 (1, 3, 10, 30 MOI) cells for 2-3 combined experiments (A). Correlation of CD activity with AdSSTR2CD dose for PC-3 (10, 30, 100, 300 MOI) and DU-145 (1, 3, 10, 30 MOI) cells for 3-4 combined experiments (B). Correlation of SSTR2 expression with CD activity after AdSSTR2CD infection of PC-3 (10, 30, 100, and 300 MOI) and DU-145 (1, 3, 10, 30 MOI) cells (C). Note that DU-145 cells are more susceptible to adenoviral infection and thus lower doses are used.

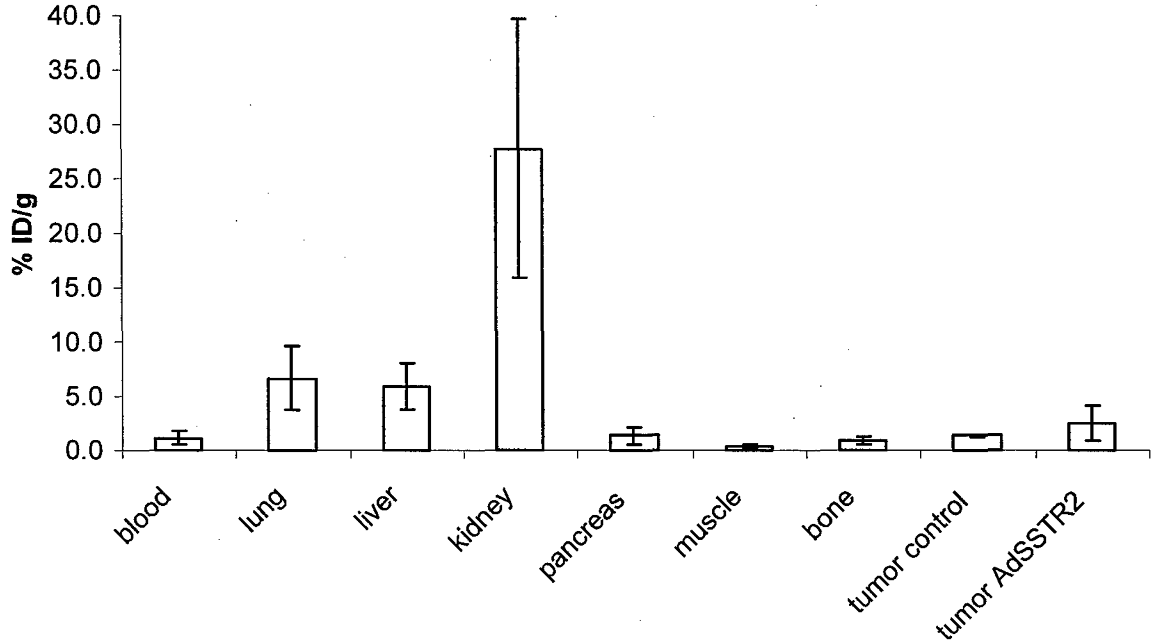
Figure 4. Michaelis-Menten plot for the conversion of 5-FC to 5-FU. The initial velocity (nmol/µg/min) for the conversion of 5-FC to 5-FU are plotted versus the concentration (mmol/L) of 5-FC ([S]) used. The saturation curve was fit to determine the V_{max}.

Figure 1



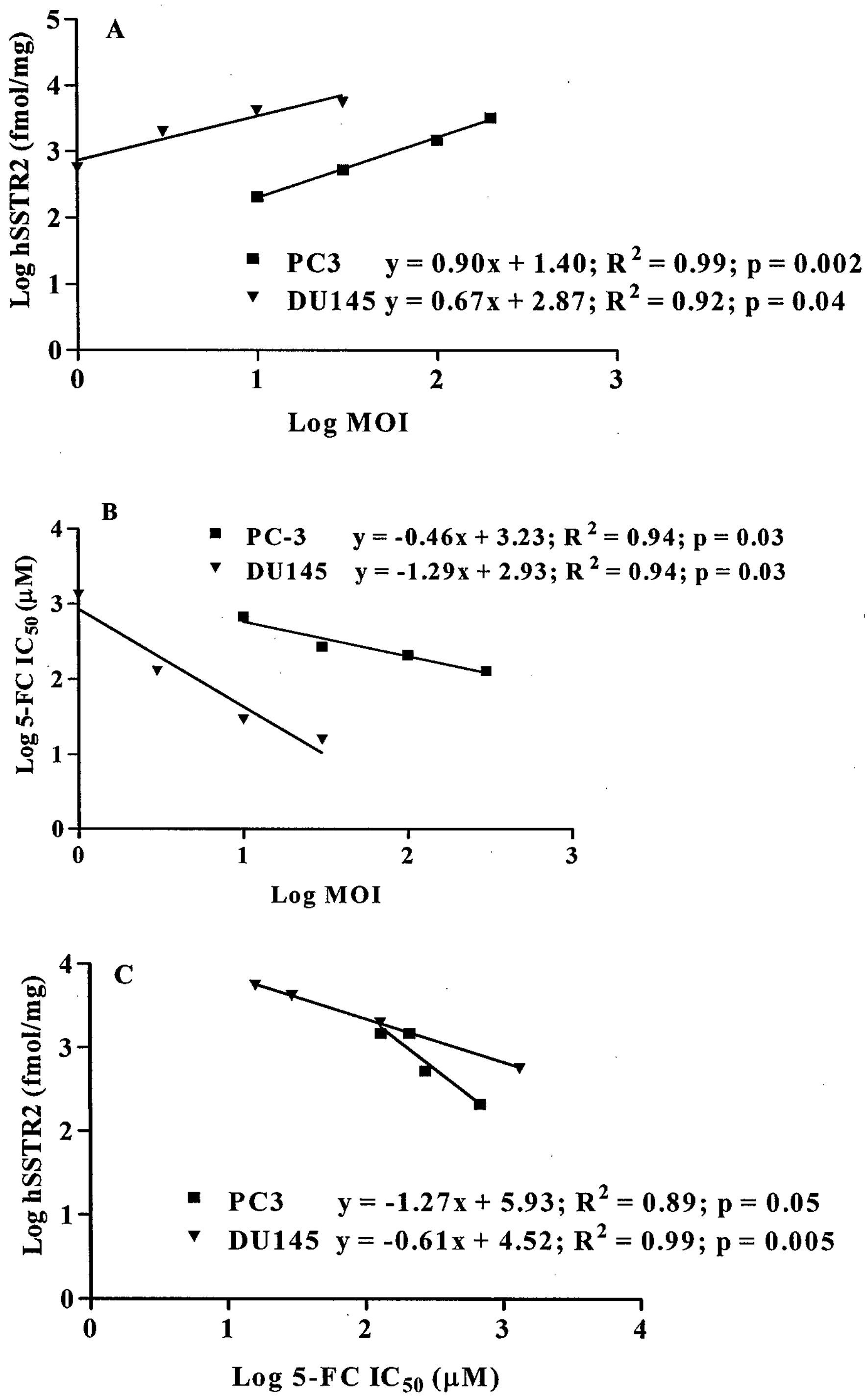
30.0
25.0
20.0
15.0
10.0
5.0
0.0
% ID/g
blood
lung
liver
kidney
pancreas
muscle
bone
tumor control
tumor AdSSTR2

Figure 2



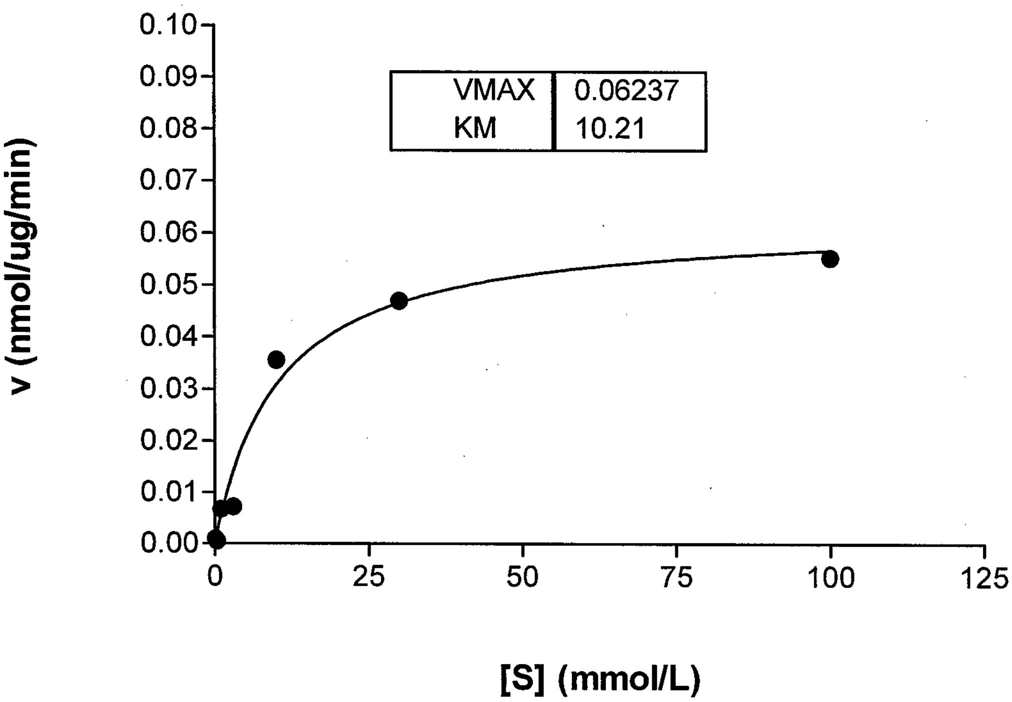
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
% ID/g
blood
lung
liver
kidney
pancreas
muscle
bone
tumor control
tumor AdSSTR2

Figure 3



A
Log hSSTR2 (fmol/mg)
Log MOI
PC3 y = 0.90x + 1.40; R^2 = 0.99; p = 0.002
DU145 y = 0.67x + 2.87; R^2 = 0.92; p = 0.04
B
Log 5-FC IC$_{50}$ (μM)
Log MOI
PC-3 y = -0.46x + 3.23; R^2 = 0.94; p = 0.03
DU145 y = -1.29x + 2.93; R^2 = 0.94; p = 0.03
C
Log hSSTR2 (fmol/mg)
Log 5-FC IC$_{50}$ (μM)
PC3 y = -1.27x + 5.93; R^2 = 0.89; p = 0.05
DU145 y = -0.61x + 4.52; R^2 = 0.99; p = 0.005

Figure 4



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0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
v (nmol/ug/min)
0
25
50
75
100
125
[S] (mmol/L)